REMARKS

This communication is responsive to the Office Action mailed July 22, 2005. The instant application was originally filed with 42 claims. In Applicant's Response dated April 5, 2004, claims 43-79 were added. Withdrawn claims 27 to 30 have been cancelled without prejudice. Applicants reserve their right to pursue the subject matter in a divisional and/or continuation application or applications. Claims 16-26, 43-61, and 65-79 are pending and stand rejected. Pending claims 62-64 have been held allowable, but are objected to as presently depending from a rejected claim.

Applicants express their appreciation for the telephone interview granted by Examiner McGarry on January 18, 2006. Applicants' representatives discussed with the Examiner the rejections noted below and the status of the application and the claims, as well as characteristics of Applicants' inventions and their ability to treat conditions as outlined the specification and claims by administration of a connexin 43 antisense polynucleotide. Remaining aspects of the interview are noted in the below responses to issues raised in the outstanding Office Action.

In this Response, various claims have been amended, and new claims 80 to 101 are added. No amendment to any claim was made for any reason relating to patentability or any outstanding rejection, and none should be construed as an admission of the propriety of any rejection, all of which are traversed in their entirety. As agreed these amendments are not made for any reason related to patentability, but for the purpose of expediting the prosecution of the subject matter of these particular claims and their focus, simplifying claim language, adjusting claim dependencies, and addressing formatting and typographical errors. As such, the new claims and the amendments to claims will not pose a burden for the Examiner, and are submitted to be in condition for allowance. A Notice of Appeal is also submitted herewith, and if required,

it is believed that the Response will remove issues for appeal. Accordingly, Applicants submit that the amendments comply with 37 C.F.R. 1.116 for an amendment after final, and respectfully request entry of the amendments presented in this response and consideration of these remarks.

Support for the adjustments to the claims is found throughout the specification and claims as originally filed. No new matter has been added. Support for new Claims 80 to 101 can be found throughout the specification, including, for example, on page 3, which refers to the use of gels and dressings.

Objections

The Patent Office objected to claims 50 and 70 as a result of certain perceived informalities. The typographical errors noted by the Examiner have been addressed, and Applicants request that the objections be withdrawn.

35 USC § 112, first paragraph: The Pending and Previous Claims Satisfy the Written Description Requirement

Several of Applicants' inventions have been confirmed to meet the written description requirement. At page 2 of the July 22, 2005 Office Action, the Patent Office acknowledged that methods using antisense constructs targeted to human connexin 43 meet the written description provisions of 35 USC § 112, first paragraph, referencing SEQ ID NO: 12 which corresponds to cDNA encoding the human species of connexin 43. The Patent Office also confirmed that methods using antisense constructs comprising SEQ ID NOS: 1, 2 and 3 meet the written description requirement.

However, claims 16-26, 43-61, and 65-79 remain rejected. As noted in MPEP § 2163.04, when an examiner bases a rejection of a claim on an allegation of lack of a written description, the examiner is to (1) identify the claim limitation not described; and (2) provide reasons why persons skilled in the art at the time the application was filed would not have recognized the description of this limitation in the disclosure of the application as filed. The Examiner appears to offer two reasons in support of his rejection, stating at pages 2-3 of the Office Action that the claims (1) encompass any species that may be a connexin 43 and thus include "a vast number of potential targets," and (2) that the specification does not specify particular antisense constructs that may target two or more different connexins. Applicants traverse this rejection. Any connexin 43 target is subject to Applicants' inventions and claims, and the number of targets that result in downregulation of connexin 43 poses no written description issue. Additionally, any connexin 43 target is subject to binding by multiple antisense polynucleotides and also poses no issue regarding written description.

Applicants' traverse is not made incomplete by the fact that various of the claims that are subject to this rejection have been altered. The following was noted to the Examiner during the January 19, 2006 telephonic interview. As will be clear from the adjustments, no claim has been amended for any reason related to patentability, including for any reason relating to the Examiner's discussion of the written description requirement. All previously pending claims fully met the requirements of 35 USC § 112, first paragraph. The language of the claims has been simplified. Amended independent claim 16 is focused on treatment of human wounds using oligonucleotides to downregulate connexin 43 for consistency in this patent with other claims also focused on wounds and/or human subjects. The new language also puts claim 16 in a form more consistent with U.S. formalities, the application and original claims having been

written and first filed in a foreign country. The amendments are made without prejudice to the filing of one or more continuing applications directed to subject matter not claimed herein. Other claims, such as claims 56 and 70, have been amended to correct typographical errors. Still other claims, such as claims 25, 45, and 53, have been adjusted solely to put them in a form more consistent with U.S. formalities given the origin of the application, as noted above.

At the top of page 3, the Office Action asserts that the species of connexin 43 targeted by SEQ ID NOS: 4, 5 and 6 are unknown, and that effective antisense sequences "cannot be predicted and must be found by a trial and error process." Applicants do not understand the Examiner's reference to the species of connexin targeted by SEQ ID NOS: 4, 5 and 6. As noted in the specification, these antisense sequences are directed, respectively, to connexins 26, 31.1 and 32. The instant claims recite connexin 43. The Examiner's statement regarding "effective antisense sequences" is also not understood. There is no requirement in the patent law that every workable embodiment within a claim be recited in the specification. The law is just the opposite. *E.g., In re Smythe*, 480 F.2d 1376, (CCPA 1973)(requiring a description of every possible embodiment of a broad term would place an undue burden on patent applicants, the Patent Office, and the public of listing, reading and examining, printing and storing, descriptions of the very many structural or functional equivalents of disclosed elements or steps).

Regarding antisense technologies in particular, as noted during the January 19, 2006 telephonic interview, Applicants refer the Examiner to Example 15 in the "USPTO Synopsis of Application of Written Description Guidelines." It describes a claim to a genus of antisense molecules that inhibit the production of human protein with a known sequence. Even though there is only a single species described with a complete structure, the Guidelines state that there

is 112 support for the genus claim, in part, because the procedures for making and testing oligonucleotide fragments are conventional.

In discussing the written description support for Applicants' claims, the Examiner also references several cases, namely, *Vas-Cath Inc. v. Mahurkar*, *Fiers v. Revel*, *Amgen Inc. v. Chugai Pharmaceutical Co.*, and *University of California v. Eli Lilly and Co.* None of these cases, however, have applicability to the matter at hand. The first is directed to support provided by drawings, and the remaining three involved composition claims directed to newly discovered genes. The following discussion of the case law was noted to the Examiner during the January 19, 2006 telephonic interview.

Vas-Cath Inc. v. Mahurkar, 19 USPQ2d 111 (Fed. Cir. 1991) involved utility patents for a "double-lumen" catheter designed to allow blood to be removed from an artery, processed and returned. The issue was whether the drawings of an application for a design patent (from which the utility applications leading to the patents-in-suit claimed priority) met the written description requirement so as to entitle Mahurkar to the benefit of the filing date of the design application for his two utility patents. The district court said no, and held the patent invalid. The Federal Circuit reversed for legal error, noting that the district court itself recognized that "what Mahurkar eventually patented is exactly what the pictures in [the design application] show."

The Examiner cited *Vas-Cath* for the proposition that, in order to satisfy the written description requirement, an applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention, and that the invention, in that context, is whatever is now claimed. It is beyond question that Applicants have done so, and possession of the inventions by Applicants is clear. Each is plainly recited in the

application, and there is no basis on which to question whether Applicants' claims are fully embraced by their original description and claims.

The Examiner cited *Fiers v. Revel*, 25 USPQ2d 1601 (Fed. Cir. 1993), for the proposition that "the nucleic acid itself is required" for a "skilled artisan to envision the detailed chemical structure of the encompassed polynucleotides . . . ." That is not the holding of this case. *Fiers v. Revel* dealt with claims to DNA coding for human fibroblast interferon-beta polypeptide (ß-IF). The court rejected Revel's claim of priority to the ß-IF DNA sequence itself on the ground that he had not included the actual DNA sequence in his patent application. Precisely, the holding of *Fiers* is that, "Conception of a substance claimed *per se* without reference to a process requires conception of its structure, name, formula, or definitive chemical or physical properties" (emphasis added). 25 USPQ2d at 1605. *Fiers v. Revel* does not support the Examiner's written description rejection of Applicants' method claims.

Amgen Inc. v. Chugai Pharmaceutical Co., 18 USPQ2d 1016 (Fed. Cir. 1991) is also beside the point, not only because it also involved product claims for a newly isolated gene (a purified and isolated DNA sequence encoding human erythropoietin), but because the description requirement was not in issue. The issue in *Amgen* was whether Amgen's patent was invalid under 35 USC 102(g) over the alleged prior invention of another. Chugai argued that the invention of Fritsch was conceived prior to Amgen's invention and thereafter diligently reduced to practice. The evidence of record was such that while Fritsch's goal was to obtain the human erythropoietin gene, and he had an idea of a possible method for obtaining it, Fritsch never did isolate the gene, nor identify its structure, and furthermore, others were unsuccessful using Fritsch's approach.

The Examiner also quotes from *University of California v. Eli Lilly and Co.*, 43 USPQ2d 1398, 1404-1405 (Fed. Cir. 1997), relying on it for the proposition that the name of a cDNA is not itself a written description of that DNA. Again, the case dealt with a claim to a gene (the human insulin gene) and is not relevant to Applicants' claims. The *Lilly* court based its decision in part upon the fact that the patent did not "provide a written description of the invention of claim 5." Claim 5 was directed to a recombinant prokaryotic microorganism modified so that it contains "a nucleotide sequence having the structure of the reverse transcript of an mRNA of a [human], which mRNA encodes insulin." That nucleotide sequence had not been identified in the patent specification.

Despite the inconsequence of the *Lilly* holding, as noted in the January 19, 2006 telephonic interview, Applicants point out that their specification provides a nucleotide sequence corresponding to a full length cDNA encoding a human connexin 43 (Table 1; SEQ ID NO:12). The polynucleotide sequence also intrinsically specifies the amino acid sequence for a human connexin 43 protein which, *ipso facto*, provides support for all polynucleotides that encode this particular protein sequence. Such conversion between amino acid sequences and nucleic acid sequences is well known in the art. *See In re David Wallach*, 378 F.3d 1330, 1334 (Fed. Cir. 2004):

> Moreover, we see no reason to require a patent applicant to list every possible permutation of the nucleic acid sequences that can encode a particular protein for which the amino acid sequence is disclosed, given the fact that it is, as explained above, a routine matter to convert back and forth between an amino acid sequence and the sequences of the nucleic acid molecules that can encode it.

For these reasons alone, the cases cited by the Examiner are distinguishable and do not support a rejection of Applicants' claims.

Additionally, as further noted during the January 19, 2006 telephonic interview, connexin 43 sequences were prior published. Page 12 of the Office Action notes that arguments/evidence

that connexin sequences are known and characterized in the prior art are to be given weight in considering Applicants' arguments for withdrawal of the written description rejection. *See Chiron Corporation v. Genentech, Inc.*, 363 F.3d 1247, 1253 (Fed. Cir. 2004) (that which was known in the art at the time the application was filed is to be considered when evaluating written description under 35 USC 112, first paragraph).

As pointed out to the Examiner during the January 19, 2006 telephonic interview, human and non-human connexin 43 polynucleotide sequences were known in the art and publicly available before the instant application was filed. For example, the following species of connexin 43 were listed in the NCBI sequence database (National Center for Biology Information, publicly available online at http://www.ncbi.nlm.nih.gov/) as of the year set forth: *Rattus norvegius* (rat) (1988); *H. sapien* (human) (1990); *Mus musculus* (mouse) (1991); *X. laevis* (frog) (1993); *Bovine taurus* (cow) (1993); *S. scrofa* (pig) (1995); and *D. rerio* (zebrafish) (1998). The *Gallus gallus* (chick) connexin 43 sequence was identified in 1990. See L.S. Musil *et al.*, "Expression of the gap junction protein connexin43 in embryonic chick lens: molecular cloning, ultrastructural localization, and post-translational phosphorylation," *J Membr Biol.* **116**(2):163-75 (1990). A partial sequence for *Equus caballus* (horse) connexin 43 was available in 1998 and a full sequence deposited in 2001.[1] A full sequence for *Canis familiaris* (dog) was deposited into GenBank on November 11, 2003 by L. A. Willingham-Rocky *et al.* (Willingham-Rocky,L.A., Golding,M.C., Westhusin,M.E., Kraemer,D.C. and Burghardt,R.C. Direct

[1] The partial sequence was submitted by W.D. Day *et al.* in 1998 (Day,W.D., Burghardt, R.C. and Ing, N.H. via direct submission to GenBank on January 14, 1998; Veterinary Anatomy and Public Health, Texas A&M University, College Station, TX 77843-4458, USA). The full sequence was deposited into GenBank by R. Einspanier via direct submission on June 28, 2001 (Physiology, Technical University Munich, Weihenstephaner Berg 3, D-85354 Freising, GERMANY).

Submission to GenBank; Veterinary Anatomy and Public Health, Texas A&M University, College of Veterinary Medicine, MS 4458, College Station, TX 77843, USA).[2]

This level of identity between the sequences described above and the human connexin 43 polynucleotide sequence provided in Applicants' specification indicates that SEQ ID NO:12 is representative of a genus of connexin 43 polynucleotides, which are not "highly variant" as alleged in the Office Action at page 5. See van der Heyden *et al.*, "Connexin43 orthologues in vertebrates: phylogeny from fish to man," *Dev Genes Evol* **214**:261-266 (2004), where the authors note that "connexin43 (Cx43) is widely expressed in all vertebrate species" (Abstract) and "displays a very broad and mainly conserved expression pattern" (page 261, col. 2). Comparison of the amino acid sequences described in Figure 3 of van der Heyden *et al.* reveal that human connexin 43 protein is highly conserved in other higher mammals. For example, human (*H. sapiens*) connexin 43 has a 99.7 % sequence homology with African green monkey (*C. aethiops*), a 97.4 % sequence homology with mouse (*M. musculus*), and a 97.6 % sequence homology with rat (*R. norvegicus*). It also has 94 % sequence homology with chicken (*G. gallus*). A copy of this article is attached herewith for the convenience of the Examiner.

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[2] Published human connexin 43 and the corresponding pseudogene sequences were reportedly used by Fishman *et al.* to perform physical mapping to identify gene location on chromosomes (Fishman *et al.*; *Genomics.*; May;10(1):250-6, 1991; GenBank Accession Nos. M65188 and M65189). Published mouse connexin 43 sequence (including the 5' untranslated region) was reportedly used in a study to identify two regulatory elements within the promoter region of the mouse connexin 43 gene by Chen *et al.* (Chen *et al.*; *J Biol Chem.* Feb 24;270(8):3863-8, 1995; GenBank Accession No. MMU17892). Published sequences for the mouse connexin 43 gene, exon 2, and 5' and 3' untranslated regions were reportedly used by Sullivan *et al.* in a study directed to the structure, sequence and expression of the mouse connexin 43 gene (Sullivan *et. al.*; *Gene*; Aug 25;130(2):191-9, 1993; GenBank Accession No. L10388, NCBI Locus MUSCX43GB). A published mouse connexin 43 sequence was reportedly used by Beyer *et al.* in a study directed to evidence that the gap junction protein connexin-43 is the ATP-induced pore of mouse macrophages (Beyer *et al.*; *J Biol Chem.;* May 5; 1991; 266(13):7971-4; GenBank Accession No. M61896). A published sequence of human connexin 43 gene, exon 1, and promoter region was reportedly used in a study by Geimonen *et al.* directed to the determination of activation of protein kinase C in human uterine smooth muscle induces connexin-43 gene transcription through an AP-1 site in the promoter sequence (Geimonen *et al.*; *J Biol Chem.* Sep 27;271(39):23667-74, 1996; Genbank Accession No. HSU64573).

Additionally, for example, Applicants' note that their SEQ ID NO: 1 polynucleotide is "human", but has 100% homology with rabbit and African green monkey connexin 43's, and only one nucleotide mismatch with bovine, pig, horse, mouse, rat, and hamster connexin 43's. Their SEQ ID NO: 2 polynucleotide is "mouse", but has 100% homology with Chinese and golden hamster connexin 43's, and only one nucleotide mismatch with bovine, rat, pig, European squirrel, Russian dwarf hamster and horse connexin 43's, and only two nucleotide mismatches with human, rabbit and hedgehog connexin 43's. Their SEQ ID NO: 3 polynucleotide is "chicken", but has only two nucleotide mismatches with human, rabbit, dog, chimpanzee and horse connexin 43's.

The instant specification also provides ample guidance for identification of target connexin 43 homologues. Applicants' specification describes procedures known and useful in the art to determine sequence homology, to align sequences, and for performing statistical analysis of sequences. In addition to the fact that other connexin 43 polynucleotides were known, these procedures can be readily employed by one of skill to identify sequences other than those published sequences, or SEQ ID NO:12, that encode variants of connexin 43, and to screen antisense molecules for use in inhibiting connexin 43 expression. Following the teachings of Applicants' specification, mutated sequences, allelic variants, splice variants, and sequences having homology with SEQ ID NO:12 can be readily identified using procedures known in the art, for example by virtue of their hybridization to SEQ ID NO:12. One of skill can also use "targeted gene walking," a procedure well known in the art at the time the instant application was filed, to identify other contiguous DNA sequences of interest. See, Parker, J.D., *et al.*, "Targeted gene walking polymerase chain reaction" *Nucleic Acids Research* 19(11), p.3055-3059 (1991).

For the reasons discussed above, Applicants respectfully submit that a proper case to support rejection for lack of written description has not been established. Accordingly, Applicants request that the rejection of claims 16-26, 43-61, and 65-79 under 35 USC 112, first paragraph, as allegedly not meeting the written description requirement be reconsidered and withdrawn.

35 USC § 112, first paragraph: The Pending and Previous Claims are Enabled

At pages 5-6 of the July 22, 2005 Office Action, the Patent Office acknowledged that the application is enabling for methods using antisense constructs targeted to human connexin 43-encoding polynucleotides, referencing SEQ ID NO: 12. The Patent Office also confirmed that methods including local administration of specific connexin 43 oligonucleotides exemplified in the application also meet the enablement requirement.

However, claims 16-26, 43-61, and 65-79 remain rejected, as allegedly not meeting the enablement requirement. The Examiner states the concern that "the claimed invention is so broad as to encompass the treatment of any condition that may be associated with connexin expression." Similarly, at page 8 of the Office Action, the Examiner states that "antisense therapy is an unpredictable art, which requires specific guidance for the treatment of any particular disease." These statements are not understood. The current pending independent claims (as well as independent claims 17, 20, 24, 26 and 54 as written prior to the wording simplifications made in this Response), refer to specific uses. For example, current claim 16 refers treatment of wounds, an indication that is thoroughly exemplified in the specification. Additionally, prior and current claim 17 references treatment of neuronal cell death resulting from a neuronal insult, prior and current

claim 24 references inflammation, prior and current claim 26 references scar formation, and prior and current claim 54 references cell death. All are fully supported by the specification and Examples therein.

Applicants traverse this rejection. As provided in MPEP 2164.04, the Patent Office has the initial burden of giving reasons supporting any assertion that a claim is not enabled. Applicants submit that this has not been done. As noted during the January 19, 2006 telephonic interview, in the main, the Office Action cites certain publications in support of an assertion that antisense is unpredictable. Applicants submit that these are not pertinent, however, to the patentability of their inventions. Theoretical reasons why some antisense compounds might not be effective in some subsequent clinical trials are not relevant where objective evidence of predictability has been shown. The instant application provides experimental evidence that the claimed antisense methods are effective in reducing expression of connexins. Importantly, this reduction in connexin 43 expression correlates with beneficial results, including for the uses claimed herein.[3]

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[3] Applicants' specification provides working examples of methods of using the claimed antisense compounds. In Experiment 1, antisense oligodeoxynucleotides (ODNs) were applied as a topical gel to chick embryos and ere shown to reduce expression of connexin within 2 hours, and up to 48 hours in some tissues. Control ODNs, on the other hand, had no effect on connexin protein expression. Experiment 2 shows the use of connexin 43 antisense ODNs *in vivo* to prevent the spread of lesions in a mammalian brain, resulting in lesions up to 50% smaller in cross sectional area as compared to untreated animals. Experiment 3 shows the ability to lessen spinal cord lesions on treatment with anti-connexin 43 ODNs. Rat models for spinal cord injuries are recognized in the art. See, for example, Holtz and Gerdin, *Journal of Neurotrauma*, 8(4):239-45 (1991); Holtz, *et al.*, "Neuropathological changes and neurological function after spinal cord compression in the rat," *Journal of Neurotrauma*, 7(3):155-67 (1990). Experiment 4 illustrates the *in vivo* treatment of incision wounds with anti-connexin 43 ODNs, showing that those treated with antisense had smaller scabs, less prominent scarring, and a more normalized hair growth pattern. A reduction in the inflammation of the wounds was also reported. The animal model system utilized in Example 4 is well established in the literature. See Martin P. *et al.*, "Wound healing in the PU.1 null mouse – tissue repair is not dependent on inflammatory cells," *Current Biology*. 13(13):1122-8 (2003); Reynolds and Fitzgerald, "Long-term sensory hyperinnervation following neonatal skin wounds," *Journal of Comparative Neurology* 358(4):487-98 (1995); and De Lima, *et al.*, "Sensory hyperinnervation after neonatal skin wounding: effect of bupivacaine sciatic nerve block," *British Journal of Anaesthesia*. 83(4):662-4 (1999).

As also noted in the telephonic interview, the publications cited by the Examiner as evidence of unpredictability fail to provide any basis to doubt that the activity stated and demonstrated by Applicants. The instant claims cover relevant uses of antisense polynucleotides (including ODNs, *etc.*) that target connexin 43, and the data provided in the specification fully supports the use of such antisense compounds. Accordingly, Applicants respectfully submit that any concern based on the supposed unpredictability of antisense approaches discussed in the cited publications is unwarranted.

For example, as Applicants also noted during the January 19, 2006 telephonic interview, the Jen article cited by the Examiner references reports on "modest" clinical effects in certain antisense drug trials and concludes that "the efficient clinical translation of the antisense strategy has proven elusive."[4] However, the point is not made. First, the article reports positively on safety. Second, whether therapeutic effects in one or more other antisense trials were modest or robust is irrelevant to the patentability of those therapies, and bear no relation whatever to Applicants' inventions. There is no basis to discuss the patentability of Applicants' inventions with respect to clinical investigation of some other therapies. In any event, the article does report clinically positive results and, while successful clinical trials are not required for patentability, to conclude that all antisense compounds have only modest clinical effects, and are therefore unpatentable, is unwarranted from the limited trials purported to have been examined and, more importantly, is contrary to law. See MPEP § 2107.03, part IV, MPEP § 2107.03, part V).[5]

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[4] The Abstract states, "Given the state of the art, it is perhaps not surprising that effective and efficient clinical translation of the antisense strategy has proven elusive. While a number of phase I/II trials employing ONs have been reported [109-116], virtually all have been characterized by a lack of toxicity but only modest clinical effects." *Stem Cells*, 18:307-319 (2000), page 515 (emphasis added).

[5] "Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials. There is no decisional law that requires an applicant to provide data from human clinical trials to establish utility for an invention related to treatment of human disorders (see *In re Isaacs*, 347 F.2d 889, 146 WSPQ

Applicants also note that citations must be read as a whole, and that indications of the viability of antisense within these references cannot be ignored. The Jen publication reports that mRNA targeting for cancer-specific therapies is "more viable than ever."[6] It has recently been reported that there has been a rapid increase in antisense molecules progressing past Phase I, II, and III clinical trials. Aboul-Fadl, *Curr. Med. Chem.*, 12(19):2193-214 (2005).[7] At least one antisense compound has been approved for use by the FDA. VITRAVENE® (fomivirsen) treats a condition called cytomegalovirus (CMV) retinitis in people with AIDS.

Applicants respectfully submit that a proper case to support rejection for lack of enablement has not been established, and request that the rejection of claims 16-26, 43-61, and 65-79 under 35 USC 112, first paragraph, as allegedly not meeting the enablement requirement be reconsidered and withdrawn.

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193 (CCPA 1963); *In re Langer*, 503 F.2d 1380, 183 USPQ 288 (CCPA 1974)), even with respect to situations where no art-recognized animal models existed for the human disease encompassed by the claims." (MPEP § 2107.03, part IV. Human Clinical Data).

"The Office must confine its review of patent applications to the statutory requirements of the patent law. Other agencies of the government have been assigned the responsibility of ensuring conformance to standards established by statute for the advertisement, use, sale or distribution of drugs. The FDA pursues a two-prong test to provide approval for testing." (MPEP § 2107.03, part V. Safety and Efficacy Consideration)

[6] The Abstract states, "Although mRNA targeting is impeccable in theory, many additional considerations must be taken into account in applying these strategies in living cells including mRNA site selection, drug delivery and intracellular localization of the antisense agent. Nevertheless, the ongoing revolution in cell and molecular biology, combined with advances in the emerging disciplines of genomic and informatics, has made the concept of nontoxic, cancer-specific therapies more viable then ever and continues to drive interest in this filed." *Stem Cells*, 18:307-319 (2000) (emphasis added).

[7] The Abstract states, "The use of antisense oligonucleotides as therapeutic agents has generated considerable enthusiasm in the research and medical community. Antisense oligonucleotides as therapeutic agents were proposed as far back as in the 1970s when the antisense strategy was initially developed. Nonetheless, it has taken almost a quarter of a century for this potential to be realized. The principle of antisense technology is the sequence-specific binding of an antisense oligonucleotide to target mRNA, resulting in the prevention of gene translation. The specificity of hybridization by Watson-Crick base pairing make antisense oligonucleotides attractive as tools for targeted validation and functionalization, and as therapeutics to selectively modulate the expression of genes involved in the pathogenesis of diseases. The last few years have seen a rapid increase in the number of antisense molecules progressing past Phase I, II and III clinical trials. This review outlines technology, its development and recent potential therapeutic applications" (emphasis added).

As previously indicated, several of the claims that are subject to this rejection have been altered. However, also as noted, no claim has been amended for any reason related to patentability, including for any reason relating to the Examiner's discussion relating to enablement. All previously pending claims fully met the enablement requirement.

CONCLUSION

For the reasons described and supported above, Applicants respectfully submit that all pending claims are now in condition for allowance. That said, if the Examiner has any remaining questions, he is encouraged to telephone the undersigned at (619) 744-2240 so that they may be promptly resolved.

Respectfully submitted,

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Dev Genes Evol (2004) 214:261–266
DOI 10.1007/s00427-004-0403-7

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Connexin43 orthologues in vertebrates: phylogeny from fish to man

Received: 5 February 2004 / Accepted: 7 March 2004 / Published online: 9 April 2004

Abstract The gap junction protein connexin43 (Cx43) is widely expressed in all vertebrate species; however, in ventricular myocardium, Cx43 expression is restricted to mammalian species only, where it provides the molecular correlate for both electrical conduction and synchronization of the repolarization process. The evolutionarily "late" appearance of Cx43 in the heart suggests physiological adaptation to euthermia with its concomitant demands related to increased cardiovascular output. We tested to what extent mammalian Cx43 differs from that of non-mammalian vertebrates and whether Cx43 from hibernating species contains specific sequence characteristics which could be attributed to their non-isothermal life cycle. We cloned the complete coding region of Cx43 from the African green monkey, European hedgehog (hibernator), Russian dwarf hamster, rabbit, European ground squirrel (hibernator) and pig. After sequencing, these were compared to 12 full-length Cx43 sequences present in GenBank (3 fish, 2 frogs, chicken and 6 mammals amongst which there was one other hibernator). Overall identity ranged from 68.7% to 97.7% at the nucleotide level and from 71.6% to 99.7% at the amino acid level. The phylogeny of Cx43 mirrors the general phylogenetic histories of the investigated species to a large extent. From 382 amino acids there were only 6 specific for mammals. There were no substitutions specific for hibernators. In conclusion, mammalian Cx43 is characterized by 6 specific amino acids, and no obvious differences between non-hibernating and hibernating mammals were observed.

Keywords Gap junction · Connexin · Sequence · Evolution

Edited by D. Tautz

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Introduction

Gap junctions provide the path for direct exchange of small molecules and ions (and current) between adjacent cells. They are built of innexins as pore-forming units in invertebrates (Phelan and Starich 2001) and of connexins in vertebrates (Goodenough and Paul 2003) and whereas their topology is similar, they present no sequence homology. In vertebrates, six connexins associate to form one hemichannel (connexon), and subsequent docking of two connexons in adjacent cell membranes results in the formation of a gap junction. To date, 21 different connexin isoforms have been described in the human genome (Willecke et al. 2002; Söhl and Willecke 2003). Of these, connexin43 (Cx43) displays a very broad and mainly conserved expression pattern in vertebrate species, i.e. in the lens epithelium of frogs (van der Heyden et al. 2001), chicken (Musil et al. 1990) and rat (Beyer et al. 1989). In the working myocardium, however, Cx43 expression is restricted to mammalian species (Becker et al. 1998). In the mammalian heart, Cx43 is vital for both conduction of the ventricular action potential and synchronization of the ventricular repolarization process.

The hibernator's heart carries several intriguing enigmas with potential relevance to future understanding of arrhythmogenesis. The heart of a hibernating animal remains electrically and mechanically active at temperatures which are incompatible with life in other mammals either due to asystole or to induction of ventricular fibrillation (Johanssen 1967). This suggests specialization during evolution with respect to membrane currents relevant for ventricular repolarization including the synchronization of this process mediated by gap junctions.

Table 1 Species abbreviations and GenBank accession numbers of connexin43 molecules described in this study

Abbr	Species	Common name	GenBank accession number
Bt	*Bos taurus*	Bovine	J05535
Ca	*Cercopithecus aethiops*	African green monkey/COS	AY382588, this study
Cc	*Cyprinus carpio*	Common carp	AY008286
Cg	*Cricetulus griseus*	Chinese dwarf hamster	AY206456
Da	*Danio aequipinnatus*	Giant danio	AF067407
Dr	*Danio rerio*	Zebrafish	AF035481
Ee[a]	*Erinaceus europaeus*	European hedgehog	AY382589, this study
Gg	*Gallus gallus*	Chicken	M29003
Hs	*Homo sapiens*	Human	M65188
Ma[a]	*Mesocricetus auratus*	Syrian hamster	AY206455
Mm	*Mus musculus*	Mouse	X61576
Oc	*Oryctolagus cuniculus*	Rabbit	AY382590, this study
Ps	*Phodopus sungorus*	Russian dwarf hamster	AY382591, this study
Rn	*Rattus norvegicus*	Norwegian rat	X06656
Sc[a]	*Spermophilus citellus*	European ground squirrel	AY382592, this study
Ss	*Sus scrofa*	Pig	AY382593, this study
Xl	*Xenopus laevis*	African clawed frog	X17243
Xt	*Xenopus tropicalis*	West-African clawed frog	AY043270

[a] Hibernator

The evolutionarily "late" appearance of Cx43 in the myocardium may point to physiological adaptation to euthermia with its concomitant demands related to a more intense energy metabolism and increased cardiovascular output. Thus, we questioned (1) which amino acids distinguish mammalian Cx43 from other vertebrate Cx43 proteins and whether (2) hibernating species display specific sequence characteristics within the Cx43 protein as a possible evolutionary adaptation to their non-isothermal life cycle. To this end we cloned the full-length coding region of Cx43 from 6 mammalian species. These were compared to 12 full-length Cx43 sequences from GenBank, including fish, amphibians, bird and other mammals. Our analysis included three hibernating species (European hedgehog, European ground squirrel and Syrian hamster).

Materials and methods

Genomic DNA was isolated by incubating tissue samples (Ee, Oc, Ps, Sc, Ss, all muscle; Ca, cultured cells) in lysis buffer (100 mM Tris/HCl (pH 8.0), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 500 μg/ml proteinase K) for 16 h at 56°C. The supernatant was phenol/chloroform extracted twice and genomic DNA was precipitated using ethanol. Subsequently, Cx43 was amplified using a mix of two sense and three antisense primers (sense ATGGGTGACTGGAGCGCCTT and ATGGGTGACTGGAGTGCCTT, antisense ATCTCCAGGTCATCAGGCCG, ATCTCTAGGTCATCAGGCCG and ATCTCCAAATCATCAGGTCG) using an annealing temperature of 56°C and 30–35 amplification cycles. The PCR products were analyzed and isolated from 1% agarose/TAE ethidium bromide stained gels using JetSorb (Genomed) and ligated into pGEM-T-easy (Promega). Cx43 was subsequently sequenced using universal Sp6 and T7 primers and Cx43-specific internal primers. Cloning of Cx43 from each species was performed twice, independently. Sequencing of both clones raised identical results.

Alignment and phylogenetic tree construction of new and full-length Cx43 sequences from GenBank was performed with Lasergene software (DNASTAR, Madison, Wis.), operating with ClustalW and Joint Neighbour algorithms (Saitou and Nei 1987; Higgins and Sharp 1990).

Results and discussion

To isolate Cx43 sequences from various species, we aligned nucleotide sequences of mouse, rat and human Cx43 and subsequently designed a primer set consisting of two sense and three antisense oligonucleotides encompassing the entire protein coding region. To test the ability to amplify novel Cx43 sequences, PCR was performed on genomic DNA from human (Hs), mouse (Mm), Russian dwarf hamster (Ps) and pig (Ss; see also Table 1). Figure 1a shows amplification of a PCR product of ~1,150 bp from all templates. Digestion with *Eco*RI (Fig. 1b) and *Sac*I (Fig. 1c) gave the predicted results for human and mouse PCR products and identical-sized fragments for Russian dwarf hamster and pig. These results demonstrate the ability of this primer set to amplify Cx43 from several different mammalian species.

Subsequently, Cx43 was amplified and cloned from African green monkey (Ca), European hedgehog (Ee), European ground squirrel (Sc), pig, Russian dwarf hamster and rabbit (Oc; see Table 1 for GenBank accession numbers). Following sequencing, alignments were performed including 12 previously described Cx43 sequences. Percentages of identity at the nucleotide level are between 68.7% (giant danio vs West African clawed frog: Da vs Xt) and 97.7% (Hs vs Ca). Identity percentages are higher at amino acid level and are between 71.6% (Da vs Xl/Xt) and 99.7% (Syrian hamster vs Russian dwarf hamster: Ma vs Ps). Figure 2 shows the result of nucleotide phylogenetic analysis. This analysis yields a clear distinction between the classes of mammals, fish, amphibia and birds and also clearly defined relationships within the classes (i.e. order of primates or suborder of hamsters).

Figure 3 reveals two main regions of dissimilarity in amino acid alignment between the Cx43 from various species. The first region is located in the intracellular loop, which is located between transmembrane domains 2 and 3; the second one follows the fourth transmembrane

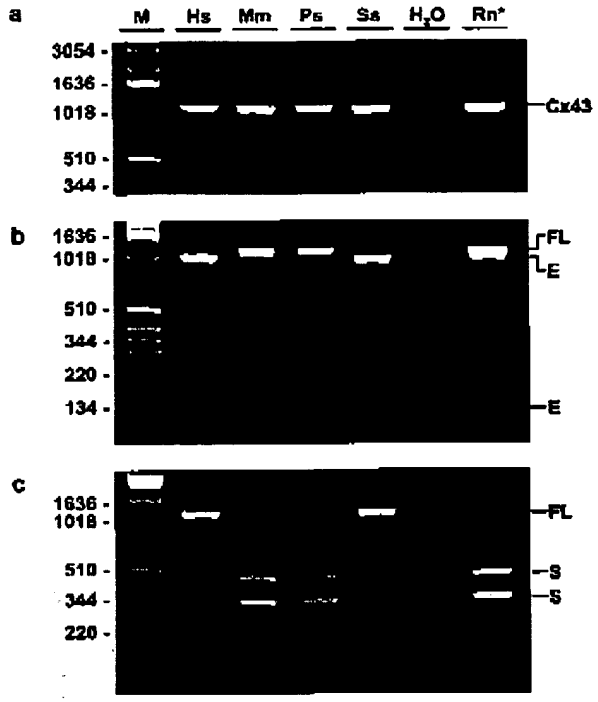

Fig. 1a–c Amplification of Cx43 from genomic DNA by PCR. a Full length product; b, c PCR product digested with *Eco*RI or *Sac*I, respectively. Position of the marker (*M*) is indicated at the left (base pairs). Species abbreviations are as in Table 1. *Lane Rn** Positive control using rat Cx43 cDNA in pBluescript vector, *H_2O* negative control; *FL* full length product, *E EcoRI* digestion product, *S SacI* digestion product

region and is thus located in the intracellular C-terminus. Currently, many residues within Cx43 have been linked to a certain function. The six cysteine residues within the two extracellular loops, which are found in all connexin protein family members, have been implicated in intercellular docking of connexons (Dahl et al. 1992). These residues are conserved between all species. Tyrosine 265 has been mapped as a Src phosphorylation site (Swenson et al. 1990) and is conserved between all species. Of the three potential PKC phosphorylation sites in the C-terminus (Kanemitsu and Lau 1993; Sáez et al. 1997; Lampe et al. 2000), S365 and S369 are conserved between all species, and hence are probably the most important. In rat, the charged amino acids R243 and D245 are implicated in membrane potential dependency of the gap junction channel (Revilla et al. 2000). Of these sites, R243 is changed into the positively charged amino acid K in many other species, indicating the importance of a positive charge at this position. The negatively charged D at 245 is changed to N in fishes only, and is conserved in all other species. Upon intracellular acidification, Cx43-based gap junctions will close (Morley et al. 1997). It is proposed that this so-called pH dependent gating results from intramolecular interactions of the C-terminus with other parts of the protein. The conserved H95 appears to play a role (Ek et al. 1994), and more recently other regions have been mapped. In the intracellular loop, two short domains are involved (rCx43 N122-L127 and I139-G143) of which the second one is well conserved (Duffy et al. 2002).

Many mutations in connexins have been linked to specific diseases (Söhl and Willecke 2003). Most of the 24 Cx43 mutations found thus far in humans, which very often result in a pleiotropic phenotype (Paznekas et al. 2002), are located at very conserved sites suggesting a general role in gap junction function, rather than a tissue-specific function. Of the four single nucleotide polymorphisms presented in the NCBI database which result in amino acid substitutions (R148Q, A168T, R202C, T204 M) the last two are conserved between all species. A168 is conserved in mammals and chicken. Interestingly, R148 is a conserved charge at this position, being K in fish and R in all other species, and thus could have a physiological role. Mutational analysis of these sites and/

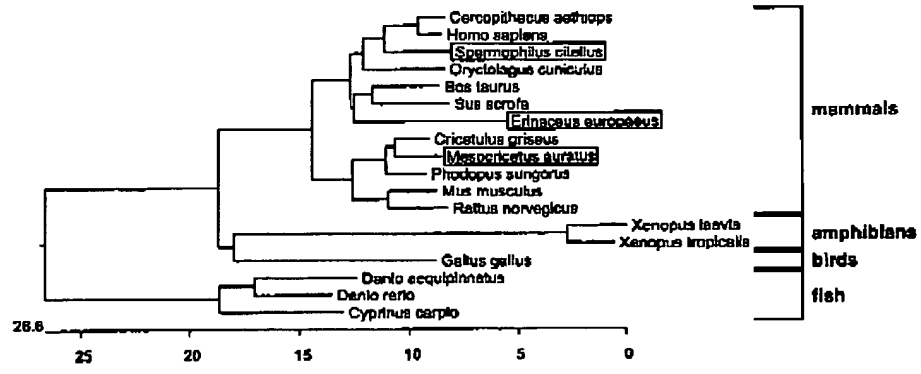

Fig. 2 Cladogram of Cx43 sequences. The nucleotide sequences of the protein coding region of 18 Cx43s from fish, birds, amphibians and mammals were analyzed using the Clustal method of the Megalign program of the Lasergene software package DNASTAR. *The scale beneath the tree* measures the evolutionary distance between the sequences, and *units* indicate the number of substitution events. Hibernating species are *boxed*

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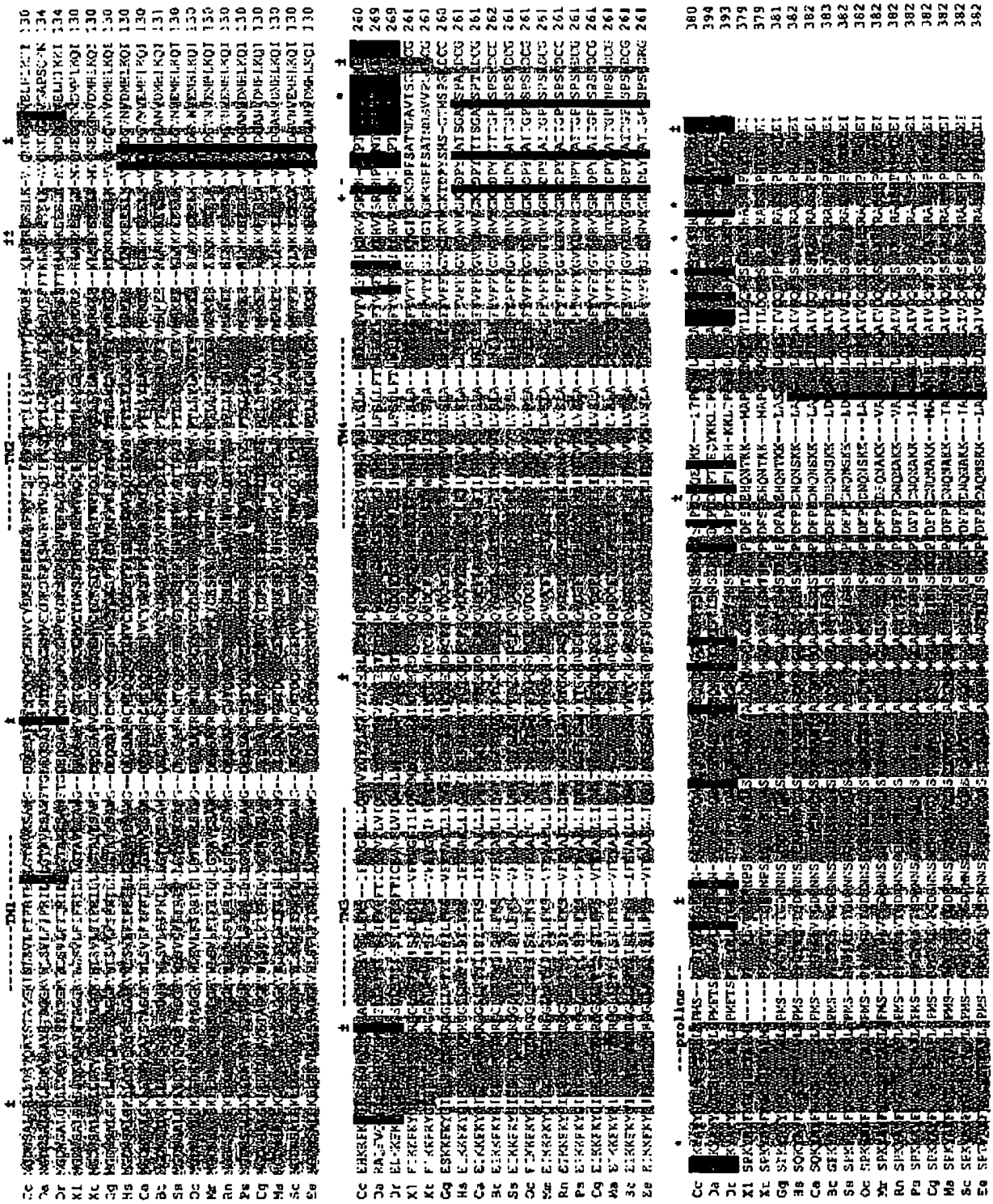

or genetic screening on diverse patient groups may result in identifying a role for these amino acids. Many amino acid changes demarcate the border between fish and other classes of the vertebrates (i.e. V36 vs L36), or between fish/amphibians and the other classes (i.e. R9 vs K9). Only six mammalian specific amino acid substitutions were observed. These substitutions demarcate the evolutionary border between the mammals and fish/amphibians/birds (with and without connexin43 in the ventricular working myocardium). These sites are shaded red (Fig. 3 Hs: A116, T118, S244, H248, L254, A349); three of these (T118, S244 and H248) are of particular interest because they may be phosphorylated (T/S) or charged (H). Interestingly, S244 is positioned within the region of the proposed membrane potential voltage sensor (Revilla et al. 2000). No relationship was found between hibernation and Cx43 coding sequences. With such a limited amount of mammalian-specific amino acids, it is not a surprise that there are no hibernator-specific amino acids (European hedgehog, European ground squirrel, Syrian hamster). On the other hand, pleiotropy might have a role as well (Griswold and Whitlock 2003). Since Cx43 is broadly expressed in many different tissues and organs, Cx43 amino acid substitutions that are beneficial for cardiac performance during hibernation might be deleterious in other organs, and therefore selected against. We suggest that in the working myocardium Cx43 serves as a gross channel of which the intrinsic properties do not allow the required fine-tuning of conduction velocity and repolarization in specific species such as hibernators. We suggest that other forms of regulation, i.e. transcriptional and post-translational regulation of Cx43 expression, or direct interactions with other proteins (reviewed in Giepmans 2004) are more important in encountering the physiological needs of a given species under specific conditions.

Acknowledgements We thank Dr. R.H. Henning and Dr. L.E. Deelman (Groningen University, the Netherlands). Dr. J. Seddon (Uppsala University, Sweden) and Dr. G. Hewitt (University of East Anglia, United Kingdom). M.C.J. Mutsaers (Super de Boer, Princehage). and P. Vrolijk (Hamstery Vrolijk, Nieuwegein) for providing tissue samples from ground squirrel, hedgehog, pig and Russian dwarf hamster, respectively. This study was supported by STW grant MKG.5942 (MvdH).

Fig. 3 Amino acid alignment of 18 full length Cx43s. Species abbreviations are as in Table 1. *TM* Transmembrane domain (there are four domains separated by two extracellular loops and one intracellular loop). *Proline* proline repeat, * experimentally confirmed phosphorylation site, + *and* − charged residues experimentally confirmed to be involved in gating, ± conserved charge, *yellow shaded* conserved between all species, *red shaded* conserved between mammals, *grey shaded* conserved between fish, *blue shaded* conserved between fish and frogs

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